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Inhibition of human leukocyte elastase by chemically and naturally oversulfated galactosaminoglycans

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Abstract

Several samples of oversulfated chondroitin and dermatan were obtained by chemical sulfation and by SAX-HPLC enrichment. The starting products and oversulfated products were tested as potential inhibitors of human leukocyte elastase, an enzyme hypothesized to be involved in the etiology of diseases such as emphysema, atherosclerosis, and rheumatoid arthritis. Chemical oversulfation (SO_3H/COOH 1.6–3.2), preferentially occurring at C-6 of galactosamine residues, was found generally to increase the inhibitory power on elastase. Chemically oversulfated galactosaminoglycans thus have potential as therapeutic agents, considering that they produce non-significant effects on the hemocoagulative system. Two naturally oversulfated dermatans sulfate (SO_3H/COOH ca. 1.2), mainly oversulfated at C-2 of iduronic acid residues, showed comparatively higher anticoagulant activity (in the HC-II mediated thrombin inhibition test).

Keywords: Chondroitin sulfate; Dermatan sulfate; Sulfation; Oversulfated galactosaminoglycans; Elastase inhibition; Anticoagulant activity

1. Introduction

Glycosaminoglycans (GAGs) bind to a variety of tissue and plasma proteins, thus contributing to the expression of several physiological and pharmacological properties [1]. Among these properties, GAGs prevent the release and the action of human

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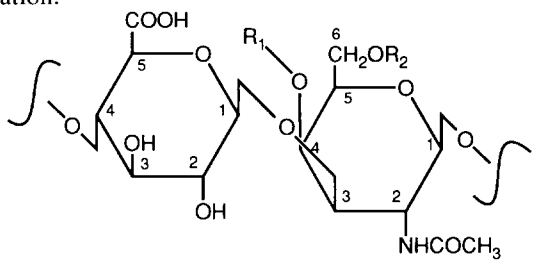
leukocyte elastase (HLE) [2], a potent serine protease found in azurophilic granules of neutrophils. A role for HLE has been suggested in the etiology of diseases such as emphysema, atherosclerosis, and rheumatoid arthritis. GAGs are therefore under active investigation as potential drugs acting through the inhibition of HLE [2–8].

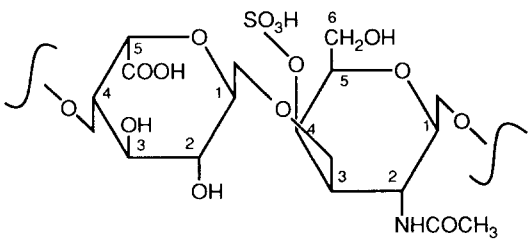
In previous structure–activity relationship studies on galactosaminoglycans, it was shown that dermatan sulfate (DS), a copolymer mainly composed of L-iduronic acid (IdoA) and *N*-acetylgalactosamine 4-sulfate (GalNAc4S), also named CSB, inhibits HLE more than chondroitin sulfate C (CSC) (a copolymer mainly composed of D-glucuronic acid (GlcA) and GalNAc6S) and much more than CSA (a copolymer mainly composed of GlcA and GalNAc4S) [5–7]. Potent inhibitors of HLE were also found among heparin and its derivatives [8].

It is known that oversulfation of GAGs (SO_3H/COOH ratio higher than in the parent compound) leads to a general increase in their protein-binding properties [1] and in particular in the inhibition of HLE [7,8]. Sulfate-rich GAGs are also known to stimulate the antihemocoagulative system [9,10].

We tested the effect of oversulfation of some preparations of CS and DS for their inhibitory power on HLE. For a more complete evaluation of their potential as drugs, the antihemocoagulative capacity of the samples was determined as a possible undesirable side-effect of oversulfation.



	R_1	R_2
α-D-GlcA*p*-(1–>3)-β-D-Gal*p*-NAc 4S Chondroitin 4-sulfate	SO_3H	H
α-D-GlcA*p*-(1–>3)-β-D-Gal*p*-NAc 6S Chondroitin 6-sulfate	H	SO_3H



α-L-IdoA*p*-(1–>3)-β-D-Gal*p*-NAc 4S
Dermatan sulfate

2. Results and discussion

Chemical oversulfation of CS and DS preparations from bovine intestinal mucosa (CS1, DS1) and from shark cartilage (CS3, CS5), gave CS2, CS4, CS6, CS7, CS8, and DS2; two naturally oversulfated fractions (DS4 and DS6) were obtained by SAX-HPLC from the two DS preparations (DS3 and DS5) from pig skin, as shown in Table 1 and described in the Experimental Section. Physico-chemical data of the samples (M_r, optical rotation, disaccharide composition, degree of sulfation (SO_3H/COOH molar ratio), i.e., sulfation index (SI)) are also reported in Table 1. Biological data (inhibition of HLE, and anticoagulant activity expressed as activated partial thromboplastin time (APTT) and as heparin cofactor-II (HC-II)-mediated inhibition of thrombin) are reported in Table 2.

The data in Tables 1 and 2 can be interpreted as follows. The interactions between GAGs and proteins are both specific and non-specific, and related to composition and sequence, charge density, molecular mass, position of the sulfate groups, type of uronic acid, and conformation of the polysaccharides [1]. These interactions are considered to occur mainly between cationic residues of the side-chains of the proteins and anionic residues of the GAGs. The main factor in favour of the non-specific protein-binding ability appears to be charge density, then molecular mass and conformational plasticity.

Table 1
Analytical data for natural GAGs and of their sulfation products

Lab. code	Origin	M_r (kDa)	$[\alpha]_D^{20}$ (°)	OS (%)	4S (%)	6S (%)	$2,6S_2$ (%)	$4,6S_2$ (%)	$2,4S_2$ (%)	SI [a]	Method of sulfation [b]
CS 1 [c]	Bovine	26.6	n.d. [c]				n.d.			1.08	
CS 2	CS 1	11.7	−18.5				n.d.			3.20	A
CS 3	Shark	44.4 [d]	−15.8	1.6	28.3	50.0	17.2	2.2	0.6	1.25	
CS 4	CS 3	10.1	−14				n.d.			2.85	A
CS 5	Shark	45	−14.5	2.5	28.4	50.1	15.8	2.5	0.6	1.21	
CS 6	CS 5	10.3	−13.4				n.d.			1.62	A
CS 7	CS 5	17.3	−14				n.d.			2.80	A
CS 8	CS 5	17.9	−14.3				n.d.			2.66	A
DS 1	Bovine	25.0	−60	1.5	87.2	2.4	0.2	1.3	7.4	1.25	
DS 2	DS 1	25.0	−51				n.d.			1.81	B
DS 3	Pig	18.5	−60	0.3	87.4	0.8	0.1	0.2	11.1	1.10	
DS 4	DS 3	21.8	n.d.	0.4	80.9	1.4	0.1	2.0	15.1	1.17	C
DS 5	Pig	9.0	−51.5	0.5	85.0	2.0	0.5	0.3	11.7	1.12	
DS 6	DS 5	14.0	n.d.	0.7	71.6	5.0	0.5	2.0	20.0	1.22	C

[a] SI = sulfation index (SO_3H/COOH molar ratio).
[b] Method of sulfation: (A) $ClSO_3H$ in DMF; (B) SO_3–TEA in DMF; (C) SAX-HPLC fractionation.
[c] Chondroitin A, B, and C mixture in which B is about 15%.
[d] $(M_r)_w$ as obtained by GPC, all others are $(M_r)_p$.
[e] n.d.: not determined.

Table 2
Activity data for the starting products and of the sulfation products

Lab. code	M_r (kDa)	SI [a]	Elastase inhibition (%) [b] (GAGS μg/mL)						APTT ± 10% [c] (U/mg)	HC-II relative potency [d]
			10	5	2.5	1	0.5	0.25		
CS 1	26.6	1.08	26(1)	22(2)	13(4)	5(1)	0	0	0.8	n.d. [e]
CS 2	11.7	3.20	74(5)	76(1)	74(1)	48(1)	22(1)	13(3)	16.5	0.64
CS 3	44.4	1.25	51(6)	48(8)	45(9)	25(7)	12(3)	0	0.3	0.23
CS 4	10.1	2.85	72(3)	65(4)	58(7)	32(4)	16(6)	9(2)	11.7	n.d.
CS 5	45	1.21	41(1)	39(1)	35(3)	20(7)	6(1)	0	0.4	0.27
CS 6	10.3	1.62	53(2)	49(4)	38(4)	19(6)	9(4)	0	0.3	n.d.
CS 7	17.3	2.80	68(4)	62(1)	57(3)	33(4)	16(2)	10(2)	46.2	0.56
CS 8	17.9	2.66	66(2)	66(1)	58(1)	31(6)	17(3)	9(1)	38.1	n.d.
DS 1	25.0	1.25	33(4)	28(5)	22(1)	17(2)	8(1)	0	0.3	1.00
DS 2	25.0	1.81	70(1)	74(1)	74(4)	53(4)	17(6)	6(2)	13.5	0.69
DS 3	18.5	1.10	37(1)	34(3)	27(1)	16(4)	3(1)	0	0.2	1.18
DS 4	21.8	1.17	51(7)	47(4)	36(7)	22(7)	6(1)	0	0.3	1.58
DS 5	9.0	1.12	30(4)	24(1)	10(1)	7(1)	0	0	0.3	1.07
DS 6	14.0	1.22	41(8)	36(9)	30(6)	15(7)	7(3)	0	0.3	1.89
Heparin									193.4	0.94

[a] SI = sulfation index (SO_3H/COOH molar ratio).
[b] Human leukocyte elastase inhibition (%); e.s.d.s in parentheses.
[c] Activated partial thromboplastin time in comparison with the IV International WHO Heparin standard (193.4 U/mg).
[d] HC-II-mediated inhibition of thrombin activity, measured by the colorimetric assay in comparison with DS 1 from bovine mucose corresponding to 235 ± 10 inhibition U/mg.
[e] n.d.: not determined.

For most proteins the binding-ability by GAGs of similar M_r is heparin > DS > CS [1]. This trend can be accounted for by a higher charge density in heparin, and by a higher conformational flexibility of the iduronate ring contained in DS, with respect to the glucuronate ring contained in CS [11]. The GAGs reported in this study display a wide range of combinations of these chemical and structural parameters, which will be used to analyse the results of the activity tests. Starting products and oversulfated products can be divided into three sets according to their inhibitory power on HLE (Table 2 and Fig. 1): set A, including CS2, DS2, CS4, CS7, and CS8, having an interpolated $IC_{50} < 2.5$ μg/mL; set B, including CS3, CS6, and DS4, having an interpolated IC_{50} of 5–10 μg/mL; and set C, including the rest, having an interpolated $IC_{50} \gg 10$ μg/mL. The most active products (set A) are those having the highest charge density, primarily CS2 and DS2, but also CS4, CS7, and CS8. Among these, DS2 has a SI of only 1.85, but it has an M_r of 25.0 kDa and is a DS-type GAG. The others have a higher SI, in the range 2.66–3.20, but have lower M_r, in the range 10.1–17.9 kDa, and are CS-type GAGs. As for the position of the additional sulfate groups, the regioselectivity of chemical sulfation is in the order C-6 of GalNAc then C-2 or C-3 of uronic acid, and C-4 of GalNAc [12]. Sulfation in DS2 occurred mainly at C-6, as shown in the ^{13}C NMR spectra by the decrease of the signal at about 64 ppm (C-6 (OH)) and

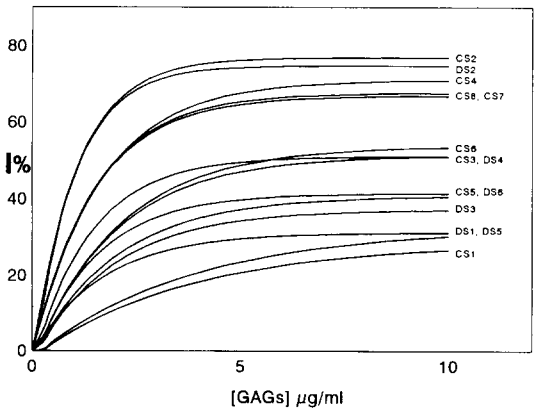


Fig. 1. Trend of HLE inhibition (%) by different GAGs at various concentrations, based on data reported in Table 1.

the increase of the signals at 70 ppm (C-6(S)). In the other compounds of set A, sulfation must have occurred also at the other available positions. Among the products in set B, CS6 has SI 1.62, M_r 10.3 kDa, and CS3 has SI 1.25, M_r 44.4 kDa. These are CS-type GAGs. DS4 is a naturally oversulfated GAG, with SI 1.17, M_r 21.8, and is a DS-type GAG, rich in C-2 sulfate. Products in set B, displaying combinations of averagely lower charge density and/or lower M_r, are less active than products in set A. Also for the products in set C, the least active, activity appears to be influenced by the same combinations of parameters. CS5 and DS6 could also be considered as a subset of set B.

It can be concluded that oversulfation, equivalent to an increase in charge density, is the main factor determining the increase in the inhibitory activity found in all tested GAGs on HLE. High charge density can compensate for a decrease in M_r, as occurred in sulfation of CS samples under strong acidic conditions ((A) in Experimental).

In view of the possibility that some of these compounds have potential as HLE inhibiting drugs, it was also verified whether oversulfation caused a potentiation in the antihemocoagulative activity of the GAGs under study, as found in similar compounds [13]. The APTT and HC-II-mediated thrombin inhibition were measured. APTT gives an assessment of the global anticoagulant activity, whereas HC-II is an assessment of a specific ability of interaction and it is currently used to characterize DS derivatives. The results are reported in Table 2 and show that the anticoagulant activity of the above-mentioned chemically oversulfated GAGs has only increased to a maximum of 46 U/mg (CS7) for APTT (heparin 193.4 IU/mg). It is worth noting that, from comparison of the APTT values found for CS2 and CS4 with those found for CS7 and CS8, the anticoagulant activity of these compounds is more dependent on the M_r than the antielastase activity. As for HC-II, its activity was increased to 0.64 U/mg in CS (CS2) and decreased to 0.69 U/mg in DS (DS2), (DS1 = 1; heparin = 0.94). In apparent

contrast, the HC-II relative potency increased up to 1.58 and 1.89 for DS4 and DS6, the two chromatographically enriched fractions, despite the low level of oversulfation. In fact, the analysis of the disaccharide composition showed that DS4 and DS6 contain a much larger amount of IdoA2S sequences than the parent compounds DS3 and DS5. This particular feature is especially effective in promoting the HC-II-mediated antihemocoagulative activity in these compounds [14], while the APTT activity, roughly correlated to charge density, did not increase.

In conclusion, oversulfated galactosaminoglycans can be considered potential therapeutic agents active as inhibitors of HLE, in particular chemically oversulfated galactosaminoglycans, which proved to be devoid of side-effects on the hemocoagulative system. Sulfation most probably increased the non-specific binding ability and led in general to products more active than the starting compounds, in a few cases with a net increase in activity, the most active ones (CS2 and DS2) having IC_{50} ca. 1 μg/mL. However, sulfation at specific positions can promote specific binding, and affect some activities more than others. In fact, an excess of IdoA2S residues in DS increases its activity on the hemocoagulative system more than on HLE.

3. Experimental

Materials and sulfation.—CS preparations were from bovine intestinal mucosa (CS1) and from shark cartilage (CS3 and CS5). DS was from bovine intestinal mucosa (DS1) and from pig skin (DS3 and DS5). All samples were supplied by OPOCRIN R and D Laboratories. The samples proved free from heparin contamination by electrophoresis on Titan III plates (Helena Labs, Beaumont). Their chemical oversulfation was carried out under rather drastic conditions (A) with chlorosulfonic acid [15] or under milder conditions (B) with adducts of sulfur trioxide (SO_3) in aprotic solvents [16]. A solution of CS (CS1, CS3, CS5) triethylamine (TEA) salt, obtained from the sodium salt by strong anion-exchange chromatography, was concentrated by reverse osmosis and lyophilized. The resulting salt was dissolved in *N*,*N*-dimethylformamide (DMF) to which the necessary amount of chlorosulfonic acid had been previously added. After 1 h at 50°C, the reaction was interrupted and the raw product was precipitated with cold acetone, neutralized with methanolic sodium acetate, then collected by filtration. Raw polysulfated CS, dissolved in water, was treated by reverse osmosis to remove salts and low molecular mass by-products. The polysulfated CS were recovered by precipitation with methanol (to give CS2, CS4). This drastic process has the drawback of the difficulty in controlling the reaction and of a certain extent of depolymerization of the samples. The sulfation mixture obtained from CS5 was fractionated by SAX-HPLC [13] giving CS6, CS7, and CS8.

DS2 only was prepared (from DS1) following a milder process (B) with SO_3–TEA in DMF. An amount of DS-TEA salt (2.8 g) was dissolved in DMF and treated with SO_3–TEA in considerable excess (13.89 g) suspended in 50 mL of DMF for 5 h. The suspension was then treated with water, adjusted to pH 6.5 with sodium hydroxide, and filtered. The filtrate was dialyzed through 3000 Da membranes, then two volumes of

methanol were added. The precipitate, collected and dried, gave a 73% yield. This process did not result in any modification in the molecular mass.

The naturally oversulfated DS4 and DS6 were isolated from DS3 and DS5, respectively, by Fractogel 45–90 μm EMD TMAE-650 (Merck), in a preparative SAX-HPLC system (Prep Jobin Yvon), and were obtained by elution with 1.5 M NaCl, as already described for similar compounds [13].

Characterization of the GAGS.—The sulfate group distribution in natural and low-oversulfated GAGs was assessed by digestion with chondroitinase ABC (EC 4.2.2.5), followed by quantitation by SAX-HPLC as previously described [13]. The specific rotation $[\alpha]$ was measured in water, $c = 0.5\%$ (w/v), $l = 1$ dm, at 298 K, 598 nm, on a Perkin–Elmer 2441 MC polarimeter. The molecular masses were determined by GP-HPLC against M_r standard compounds on a third order polynomial calibration curve. The SO_3H/COOH ratio was determined by potentiometry with a Metrohm 682 titroprocessor, as previously described [17].

Activity measurements.—The inhibiting activity of the GAGs on HLE was measured, as previously described [6], on enzyme supplied by Serva (Heidelberg), specific activity 16–18 U/mg. About 40 mU of HLE were used per assay, run in duplicate, twice for each concentration, for each GAG. The four values were averaged and used for the calculation of the standard deviation. The HC-II-mediated inhibition of thrombin (Factor IIa) activity was measured, by the colorimetric method, in comparison with DS1, a sample of DS from bovine intestinal mucose, corresponding to 235 ± 10 inhibition U/mg, as previously described [13]. The APTT activity was measured versus the fourth WHO International Standard of Heparin by comparing b of the regression lines, according to Thomson and Poller [18].

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